B. **Remarks**

Claims 5-9, 14, 16-17, and 23-25 are presently pending. Claims 18-22 were cancelled without prejudice or disclaimer in response to the Final Rejection of May 7, 2003. Claims 1-4, 10-13 and 15 are withdrawn from consideration as directed towards the non-elected invention. Claim 5 has been amended.

Only one rejection remains in this case, the 35 USC §112, first paragraph rejection, i.e., the "written description" rejection. Applicants respectfully request reconsideration of the rejection based upon the claim amendment and the following argument. The response will concentrate on four main topics:

1) To begin, a summary of the rejections made by the Examiner are useful.

2) Then, an analysis of the case law about the written description requirements it applies to:

a. the guidelines issued by the USPTO itself on the interpretation of the written description requirements;

b. the CAFC decision ***Enzo v. Gen-Probe***; and,

c. the MPEP, as to the "description" of a micro-organism in a patent application (extracted from the ***Enzo v. Gen-Probe*** decision).

3) Third, in addition to the technical documents already on file, documents are herein provided that further demonstrate the techniques used in the present invention are art-recognized techniques for characterization of micro-organisms, in particular of viruses, and, thus, that these techniques

unambiguously prove the "possession" of the invention when the application was filed.

4) Fourth, and finally, the specification both from a quantitative- and qualitative point-of-view provides technical information and guidance that (is more than sufficient to) demonstrate "possession" of a genus of ERS strains/isolates.

The Examiner's Rejections:

In one statement, as found on page 2 of the Final Office Action of May 7, 2003, the Examiner questions whether the application provides adequate support for the broadly claimed genus of avian reoviruses. The Examiner's primary contention is that the invention may not be adequately described where an invention is described solely in terms of a method of its making coupled with its function and there is no art-recognized correlation or relationship between the structure of the invention and its function. The corollary used by the Examiner is to a situation where a biomolecule sequence is claimed. However, Applicants' claimed invention is not concerned with a biomolecule sequence, but with a complete (micro-)organism, i.e. a virus. However, the Examiner admits that possession may be shown by disclosure of drawings or structural formulas that are sufficiently detailed. Such sufficiently detailed, relevant identifying characteristics may be complete or partial structure, ..., functional characteristics when coupled with a known or disclosed correlation between function and structure. See page 3, Final Rejection of May 7, 2003. The Examiner then lists a series of examples of identifying characteristics of 'being in possession of a claimed invention' as

its nucleotide or amino acid sequence, chemical structure, binding affinity, binding specificity and molecular weight. However, Applicants claim a biomolecule. Further, and notably, , the Examiner correctly states that written the description requirement can be satisfied through disclosure of function and minimal structure when there is a well-established correlation between structure and function. Applicants have established and will establish further correlation.

On page 4 and 5 of the Final Rejection of May 7, 2003, the Examiner outlines why the present disclosure fails to provide adequate guidance and lists a series of factors:

(1) The complete nucleotide or amino acid sequence of any given avian reovirus is lacking, even for the specifically deposited isolate. However, the Federal Circuit has held otherwise. The Enzo-court came to an opposite conclusion in this respect, as will be discussed below. The Examiner concludes that the disclosure fails to identify the structure and any critical molecular determinants, that modulate the phenotypic properties of any given avian reovirus. However, Applicants contend that a plethora of art accepted phenotypic properties and determinants have been disclosed.

(2) The Examiner mistakenly is of the opinion that the virus neutralization properties (as measured in a plaque reduction assay) and the monoclonal antibody binding characteristics do not provide any information to the genotypic properties of any given isolate. Such an impression is mistaken.

(3) Further, the Examiner contends that the disclosure fails to identify any critical antigenic or immunogenic determinants and that nothing in the

disclosure would lead the skilled person to any particular isolate other than the deposited one.

(4) Lastly, the Examiner repeats a contention that the plaque reduction assay fails to provide any guidance pertaining to the molecular determinants of the virus and that the methodology described does not allow the skilled person to define the claimed product. Applicants respectfully request reconsideration.

In response to Applicants' previous arguments, the Examiner contends as follows:

(1) The Examiner contends that the serological properties (resulting from the tests used in the description) provide no further understanding of the molecular determinants (genotype) of the isolates. The Examiner submits that the (two) tests used are generic assays that do not allow a skilled artisan to (i) define a genus of genotypically/phenotypically homogeneous isolates and/or to (ii) distinguish viruses of the present invention from other viruses. However, Applicants' experimentation and characterization is an art accepted manner of identifying viruses.

The Examiner appears to want Applicants to use other properties, such as virion size, shape etc.... However, these terms are inherent and defined by Applicants use of the term "avian reovirus." The term, used in the claims, represents a multitude of (inherent) structural and functional limitations that (together with the further immunogenic property(s) mentioned in the claim), readily allows the skilled person to identify whether he is in possession of the claimed subject-matter.

(2) Although many isolates are mentioned in the description, the Examiner is of the opinion that no detailed structural or functional characteristics (such as the nucleotide sequence) of these isolates are provided. However, the law does not require a nucleotide sequence to comply with the written description requirement.

(3) According to the Examiner, the case law applied by him is relevant. However, the case law is dependent upon the fact situation and words cannot be taken from a case and stuck into a rejection. Here, the fact situation dictates that the case law be applied differently.

Case Law Analysis:

a. The USPTO written description guidelines

Example 9 of the MPEP (Hybridization)

In this example, a single cDNA sequence (SEQ NO: 1) is disclosed that is said to hybridize to other nucleic acids under stringent conditions and these nucleic acids also encode a protein that binds with a certain receptor.

The claim is directed to a genus of nucleic acids all of which must hybridize with SEQ NO: 1 under highly stringent conditions.

The guidelines reason such complies with the written description requirement using the following reasoning:

- Hybridization techniques using DNA as a probe were conventional in the art.

- A single species is disclosed that is actual reduced to practice.

- The skilled person would not expect substantial variation among species encompassed within the scope of the claims because the highly stringent conditions mentioned in the claim yield structural similar DNAs.

Thus, in conclusion, a representative number of species is disclosed, as the hybridization conditions in combination with the coding function and the level of skill and knowledge in the art adequately determine possession. Much the same as the present case.

Example 14 of the MPEP (Product by function)

A protein having an amino acid sequence as in SEQ NO: 3 is disclosed that in addition has the property to catalyze a certain reaction. Variants (at least 95% identical) of the protein are contemplated but not exemplified. Procedures for making such variants are conventional and an assay to determine the catalytic activity is described. The claim is directed to SEQ NO: 3 and its variants (at least 95% identical and having the catalytic activity).

The guidelines reason such complies with the written description requirement using the following reasoning:

- There is actual reduction to practice of the single species.

- The genus of variants do not have substantial variation as the amino acid sequence of variants is at least 95% identical and have the specified catalytic activity.

Thus, in conclusion, the single species is representative of the genus because of these limitations and of the presence of the assay.

Example 16 Antibodies

An antigen X has been isolated and characterized. The specification contemplates but does not teach in an example any antibody which specifically binds to the antigen. The general knowledge in the art is such that antibodies are structurally well characterized, e.g. antibodies contain antigen binding sites in the form of complementary (to the antigen) determining regions. The claim is directed to antibodies that are capable of binding to antigen X.

The guidelines reason such complies with the written description requirement using the following reasoning:

- Again, the level of skill and knowledge in the art (of antibodies) is deemed important, leading to the conclusion that this level was such that the production of antibodies against a well characterized antigen was conventional.
- Considering the routine methods of making antibodies, the well defined structural characteristics of antibodies, including the functional characteristics of antibody binding requirements, the skilled person would recognize that the spectrum of antibodies that bind to antigen X are disclosed as a result of the isolation of antigen X.

Thus, in conclusion, the MPEP provides guidance that once a specie is disclosed also variants of such a specie that do not substantial differ structurally from that specie are described.

b. The CAFC decision ***Enzo v. Gen-Probe***

The present case, as well as the ***Enzo v. Gen-Probe*** case, is distinguished from the Eli Lilly case (heavily relied on by the Examiner in examination of Applicants' claims). In ***Eli Lilly***, it was held that in case (a gene) material has been defined only by a statement of function or result, such statements alone did not adequately describe that material.

In contrast, Applicants invention is a claim of:

(i) the "avian reovirus" , in combination with

(ii) the immunogenic properties as defined in the claims.

Applicants contend such represents a precise definition of the claimed subject-matter as to its structural- and physical characteristics, and that this case for exactly this reason, is distinguished from the ***Eli Lilly*** case.

The ***Enzo*** Court held that:

"*It is not correct, however, that all functional descriptions of genetic material fail to meet the written description requirement*", and referring to the MPEP that the written description requirement can be met by "*showing....sufficiently detailed, relevant identifying characteristics, e.g. functional characteristics when coupled with a known or disclosed correlation between function and structure..*" (page 5, 1st full paragraph).

The ***Enzo***-court also referred to Example16 of the USPTO written description guidelines: antibodies are adequately described by means of their binding property with a certain antigen, because this binding property implies well defined structural characteristics of such antibodies. ***See Enzo Biochem Inc. v. Gen-Probe Inc.***, 63 USPQ2d 1609, 1613 (CA FC 2002).

Although the Court did not decide on the fulfillment of the written description requirement for those claims that cover a genus of nucleotide

sequences, because this is a matter of fact specific to the case, it nevertheless provided guidance how (for the District Court) to assess such a question, an such guidance applies to the USPTO:

In ***Ely Lilly*** the specification "*did not set forth any common feature possessed by members of the genus that distinguished them from other*". Notably, the ***Enzo***-court specifically refers to Example 9 of the written description guidelines that mirrors the ***Enzo***-case in that the Example 9 also concerns a genus claim to nucleic acids based on their hybridizing properties. As already outlined above, the USPTO guidelines determined that such a genus claim fulfilled the written description guidelines, because all species within the genus will be structurally similar.

In other words, the Enzo-court approaches the same question from another side: nucleotide sequences that preferentially bind to the described genomic DNA of certain deposited bacteria may also be adequately described because such sequences have a (complementary) structural relationship with the genomic DNA. ***See Id.*** at 1693-4. This reasoning is very similar to the Antibody-Antigen Example 16 in the written description guidelines.

c. The MPEP, as to how to describe a micro-organism in a patent application (interpreted in the ***Enzo v. Gen-Probe*** decision)

The ***Enzo***-court makes perfectly clear that practical difficulties are associated with describing unique biological materials in a written description and stresses both the importance and well accepted practice before the USPTO of depositing a sample of the micro-organism to satisfy the §112, 1st paragraph requirements, including the written description requirement: "..we hold that reference in the specification to a deposit in a public depository, which makes its contents accessible to the public when it is not otherwise available in written form, constitutes an adequate description of the deposited material sufficient to comply with the written description requirement of §112, 1". **See Id.** at 1613. The Enzo-court held that micro-organisms "are adequately described in the specification by their accession numbers" and that a description of a micro-organism by virtue of its genomic sequence (which would be unduly burdensome, because it would take 3000 scientists one month to sequence the genome of one micro-organism) is by no means a prerequisite for satisfying the written description requirement. ***See Id.*** Clearly, the ***Enzo***-court is of the opinion that the description of a biomolecule is something different than the description of a complex biological material, such as a micro-organism, and that the deposit of biological material is a well established manner to describe such material.

The Techniques Used by Applicants are art accepted:

The documents previously submitted and the following documents unambiguously demonstrate that a plaque reduction assay (based on virus

neutralization by antiserum) is a conventional test that demonstrates antigenic similarity between related viruses in a very specific and selective manner (see the various previous citations in the responses). Further, when compiled with a monoclonal binding panel pattern, complete disclosure is made and one of ordinary skill in the art has the invention.

The tests provide functional characteristics, i.e. (i) the neutralization of a virus by antiserum or (ii) the binding of a monoclonal antibody to the virus and, importantly, the tests correlate directly with the structure of the viruses, i.e. the presence or absence of certain antigenic determinants or epitopes on the surface of the virus. In other words, the tests provide an art-recognized correlation or relationship between the function and structure. The tests provide information on the phenotypical properties of the virus (presence/absence of certain antigenic determinants) and, hence, on the genotypical properties of the virus, i.e. the presence /absence of the corresponding coding sequences for these antigenic determinants.

Serological tests based on virus neutralization or binding with monoclonal antibodies are not just random tests, but are the tests utilized in this artfield to demonstrate (structural) similarity between species within a genus and to distinguish these species from structurally unrelated antigens/species.

The neutralization of a virus by a certain antiserum or the binding pattern of a panel of monoclonal antibodies provide an "antigenic fingerprint" of the virus, and as such structurally characterizes the species within a genus of genotypically/phenotypically homogeneous virus isolates.

The following documents further prove that the assays used by the present inventors are very appropriate to identify and characterize virus strains or isolates of the same serological/antigenic type and, thus, to define a genus of homogeneous strains or isolates (Copies are included with this response):

Estes et al. (Am J Vet Res 41, 151-152, 1980):

"*In addition, neutralization of viral infectivity with specific antiserum before assay (plaque reduction) test is shown to be a useful test for identification of species of origin of rotaviruses*" (page 151, left column).

"*The plaque assay and plaque reduction tests should become useful in distinguishing serotypes and titrating..... The ability to differentiate serotypes of rotavirus will be important for the development of vaccines for the control of rotaviral infections*" (page152, last paragraph)

Green et al. (J. Virology 62, 1819-1823, 1988):

"*In addition, the predicted serotype from sequence analysis correlated in each case with the serotype determined when the rotaviruses were examined by plaque reduction neutralization or reactivity with serotype-specific monoclonal antibodies*" (Abstract).

Kang et al. (J. Clin. Microbiol. 31, 2291-2297, 1993):

"*From the serological analyses, three different reactivity patterns were recognized by plaque reduction virus neutralization and cell culture immunofluorescence test*" (Abstract and Table 3).

To conclude, the Examiner's opinion that,

(i) the assays used by the present inventors do noting more than "merely citing a particular immunological property without a further understanding of the molecular determinants modulating that activity

fails to provide any further illumination pertaining to the genotype of any given isolate", and

(ii) Applicants are attempting to define a large genus of genotypically/phenotypically independent and distinct viruses based upon a rather generic assay,

is not supported by the wealth of publications supplied to the Examiner. In fact, as recognized in the art, these tests provide the opposite types/different types of information and, thus, for this reason are suited to demonstrate possession of a genus of immunologically homogeneous isolates of avian reoviruses.

Importantly, it is noted that also the term "avian reovirus" used in the claims, inherently, defines the claimed subject-matter in a structural manner, as the claimed virus comprises the known structural properties of an avian reovirus. For example, virion properties of reoviruses, and avian reoviruses in particular, are disclosed in the following textbook:

Virus Infections Of Birds, eds.: Mc Ferran and McNulty, Elsevier Science Publishers B.V., 1993, pages 177 and 181. (previously submitted to the Examiner).

Applicants have Possession of the Claimed Invention:

Applicants have amended Claim 5 to incorporate the limitations of the monoclonal binding pattern and the plaque reduction assay. Claim 5 now reads "[a] vaccine comprising an avian reovirus belonging to an antigenic class of avian reoviruses ERS isolates, wherein the avian reovirus is able to induce antiserum in an animal, which antiserum causes a reduction of the

plaques formed by avian reovirus ERS, a sample of which is deposited at the ECACC under accession no. 99011475, of at least 75% in a plaque reduction assay and wherein the avian reovirus positively reacts with polyclonal avian reovirus antiserum but not with monoclonal antibodies identified by accessions nos. 99011472, 99011473 and 99011474, samples of which are deposited at the ECAC, and a pharmaceutical acceptable carrier or diluent." Accordingly, Applicants have further defined their invention. However, the amendment does not limit the scope of the claim and will not effect the application of the doctrine of equivalents because such amendment was inherent in the disclosure. The ERS type isolate of an avian reovirus is identified by both the monoclonal binding pattern and the plaque reduction assay of the present invention. Accordingly, Applicants respectfully request reconsideration.

Moreover, the specification on page 6 of the present invention and Example 1A provide the method and the tools to the skilled person to isolate and identify the new ERS isolates. Further, Examples 1B-C disclose 13 identified representatives. As well, Tables 2A and B indeed prove (as outlined above) that the antiserum raised against prior art, non-ERS isolates is not able to cause plaque reduction of ERS isolates, whereas antiserum raised against an ERS isolate is able to reduce the plaques of the specifically deposited ERS isolate (ERS-1, see also page 7, last paragraph).

In addition, the (Moab) binding pattern as specified in the Claims further defines the antigenic/structural properties of the claimed ERS isolates. The Moab reaction pattern makes clear that the claimed ERS isolates:

(i) bind with polyclonal anti-avian reovirus antiserum (and with certain Moabs that apparently bind with antigenic determinants that are conserved in all avian reoviruses), but

(ii) do not bind with the three specified Moabs INT-14-11, INT-13-06 and 15-01 INT. This functional property of the claimed ERS isolates implies that ERS isolates do not have these antigenic determinants that are present on known avian reoviruses.

In conclusion, the Claims are in compliance with the USPTO's written description guidelines and the guidance provided by the ***Enzo***-Court, the skilled person would directly recognize the spectrum of claimed ERS isolates and that the inventors were in the possession of a genus of ERS isolates that do not substantially differ structurally.

III. CONCLUSION

In light of the Argument above, Applicants respectfully request reconsideration of the rejection and allowance of the case. Applicants further respectfully request that a personal interview be granted during the week of December 2nd, 2003 between Applicants' attorney and the Examiner. Please charge any required fees to deposit account 02-2334 and credit any credits.

Sincerely,

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Identification of Rotaviruses of Different Origins by the Plaque-Reduction Test

M. K. Estes, PhD, and D. Y. Graham, MD

SUMMARY

The plaque assay for the simian rotavirus SA11 was shown to be applicable to the economically important calf and porcine rotaviruses. In addition, neutralization of viral infectivity with specific antiserum before assay (plaque-reduction test) was found to be a useful test to identify the species of origin of rotaviruses.

Rotaviruses have been identified as etiologic agents in neonatal diarrhea in many mammalian species.[1] In both the cattle and the swine industries, rotavirus-induced diarrhea is a significant cause of neonatal mortality and morbidity. Research aimed at understanding the pathogenesis, epidemiology, and virology of rotavirus disease has been hampered by the lack of a rapid, sensitive, reproducible, and easy assay method for rotavirus infectivity. We have recently developed a plaque assay for the simian rotavirus SA11 which fulfills those requirements.[2] This report extends the plaque technique to the economically important calf and porcine rotaviruses. In addition, neutralization of viral infectivity with specific antiserum before assay (plaque-reduction test) is shown to be a useful test for identification of species of origin of rotaviruses.

Materials and Methods

Porcine rotavirus (OSU strain), bovine rotavirus, and rotavirus antiserum obtained after infection of gnotobiotic animals were supplied by Drs. E. Bohl (porcine)[a] and C. A. Mebus (bovine).[b] Guinea pig immune sera to bovine rotavirus were supplied by Dr. L. Spence.[3,c] Simian rotavirus SA11, originally supplied by Dr. H. H. Malherbe,[d] and hyperimmune guinea pig SA11 antisera were prepared in this laboratory as previously described.[4] The plaque assay was done as previously described.[2] Briefly, monolayers of fetal rhesus monkey kidney cells (MA104 cells) either (i) were maintained in Eagle's minimal essential medium (EMEM) without serum for 2 to 3 days before assay or (ii) were washed three times with tris-buffered saline solution to remove residual serum before inoculation of 0.1 ml of serial dilutions of viral preparations. After viral adsorption at 37 C for 90 minutes, an agar overlay was applied. The overlay medium consisted of EMEM containing 1.5% bactoagar,[e] 0.03% glutamine, 0.3% sodium bicarbonate, 0.02 mg of neutral red/ml, 100 units of penicillin/ml, and 100 μg of streptomycin/ml. In addition, the facilitators DEAE-dextran (100 μg/ml) and pancreatin were required. Pancreatin[f] was prepared by dissolving one tablet (approx 1 g) in 50 ml of distilled water. A 1:60 dilution of this stock was used in the overlay. For an alternative source of enzyme, pancreatin[g] can be used if diluted so that the final trypsin concentration in the overlay is 0.2 U/ml, as previously described.[2] Two stocks of pancreatin[g] tested required a 1:600 to 1:700 dilution to obtain this concentration. Plaques were read after 4 to 6 days' incubation at 37 C.

Neutralizaton Tests—Neutralization tests were performed by mixing equal volumes of (i) dilutions (1:10 to 1:30,000) of antiserum (previously heat-inactivated at 56 C for 30 minutes) and (ii) virus diluted to contain approximately 100 plaque-forming units (PFU) per 0.1 ml. After an incubation of 30 minutes at 37 C, 0.2-ml aliquots of the virus-antiserum mixture were inoculated onto the monolayers. Control cultures were inoculated (in parallel) with virus mixed with tris-buffered saline solution.

Results

Representative plaques observed using this assay method with simian, bovine, and porcine strains of rotavirus are shown (Fig 1). These plaques were obtained with the Nebraska calf diarrhea virus obtained from Dr. Mebus; however, other bovine rotavirus strains obtained from Dr. G. Woode (strain B14) and from Dr. Spence (strains BR486 and BR2352) also induced plaque formation. The simian rotavirus usually yielded the largest plaques; the bovine rotavirus strains induced intermediate-sized plaques; and the porcine rotavirus induced the smallest plaques.

The ready adaptation of the plaque assay to permit serologic identification of rotavirus strains is shown (Table 1). Hyperimmune SA11 antiserum prepared in guinea pigs completely neutralized plaque formation by the simian rotavirus. There was no inhibitory effect (cross-neutralization) of the simian agent by antiserum prepared against either the porcine or the bovine rotavirus. Similarly, the porcine rotavirus was easily distinguished, using potent antiporcine rotavirus serum which prevented plaque formation. Porcine rotavirus infectivity was not neutralized by antisera prepared against the bovine and the simian agents. Similarly, bovine virus antiserum prepared in guinea pigs effectively differentiated between the porcine, bovine, and simian rotavirus strains. The bovine rotavirus strain was only moderately neutralized by bovine rotavirus antiserum prepared

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[d] University of Texas Health Science Center, San Antonio, Tex.

[e] Difco Laboratories, Detroit, Mich.

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[g] Gibco, Grand Island, NY.

Fig 1—Rotaviral plaque formation in MA104 cells. Control (noninfected) monolayers (A) or monolayers were inoculated with simian, strain SA11 (B); bovine strain, neonatal calf diarrhea virus (C); or porcine, strain OSU rotaviruses (D). Cultures were photographed 6 days after inoculation.

TABLE 1—The Plaque-Reduction Test: Specific Inhibition of Plaque Formation by Neutralization of Simian, Porcine, and Bovine Rotaviruses

Rotavirus	Antisera				
	None[a] (control)	Anti-simian[b]	Anti-porcine[c]	Anti-bovine[d]	Anti-bovine[e]
Simian origin	1.8×10^7	$<10^4$	1.0×10^7	1.0×10^7	2.7×10^7
Porcine origin	5.8×10^6	3.6×10^5	$<10^2$	4.0×10^5	5.3×10^6
Bovine origin	3.2×10^6	4.0×10^5	2.3×10^5	$<10^2$	5.0×10^5

[a] Tris-buffered saline solution; [b] Hyperimmune guinea pig anti-SA11 serum, used at 1:30,000 final dilution; [c] Gnotobiotic porcine anti-porcine rotavirus serum obtained from Dr. Ed Bohl, used at 1:1,000 final dilution; [d] Immune guinea pig anti-BR 2352 obtained from Dr. L. Spence, used at 1:200 final dilution; [e] Gnotobiotic bovine anti-NCDV serum obtained from Dr. C. A. Mebus, used at 1:100 final dilution.
Data are expressed in PFU/ml.

in a gnotobiotic calf. Although the gnotobiotic calf serum did permit differentiation between the porcine, bovine, and simian rotavirus strains, the potency of that particular antiserum was lower than that produced either in the gnotobiotic pigs or in the hyperimmunized guinea pigs.

Discussion

The demonstrated ability of a plaque assay to measure the infectivity of the porcine and bovine rotaviruses should allow for more rapid advancement in our knowledge of the basic biology of these economically important viruses. This rapid and reliable assay, in combination with neutralizing antiserum, can identify rotavirus strains in a plaque-reduction test. Application of this test, using well-characterized and high-quality antiserum, will facilitate identification of rotavirus strains in laboratories working with more than one rotaviral agent. The success of this test, however, is dependent on the production of high-titer antisera. Due to the antiviral activity (which appears to be antiproteolytic activity[4,b]) present in all sera at concentrations as low as 2%, specific reactions may require antisera which can be diluted at least 1:100. Although the specificity of the test is dependent upon careful characterization of antisera, it is nevertheless simpler than techniques presently available to distinguish strains, such as polyacrylamide gel electrophoresis patterns of viral RNA,[5] the blocking ELISA test,[6] hemagglutination-inhibition tests,[3] or immunofluorescence neutralization tests.[7,8]

The plaque assay and plaque-reduction tests should become useful in distinguishing serotypes and titrating infectivity of new isolates that have been adapted to growth in tissue culture with the help of proteolytic enzymes.[9–11] The ability to differentiate serotypes of rotavirus will be important for the development of vaccines for the control of rotaviral infections. In addition, the ability to plaque different rotaviral serotypes under the same conditions and in the same cell lines will greatly simplify attempts to study the replication and genetics of this new viral group.

[b] Unpublished data: 1979.

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Prediction of Human Rotavirus Serotype by Nucleotide Sequence Analysis of the VP7 Protein Gene

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Human rotavirus field isolates were characterized by direct sequence analysis of the gene encoding the serotype-specific major neutralization protein (VP7). Single-stranded RNA transcripts were prepared from virus particles obtained directly from stool specimens or after two or three passages in MA-104 cells. Two regions of the gene (nucleotides 307 through 351 and 670 through 711) which had previously been shown to contain regions of sequence divergence among rotavirus serotypes were sequenced by the dideoxynucleotide method with two different synthetic oligonucleotide primers. The resulting nucleotide sequences were compared with the corresponding sequences from rotaviruses of known serotype (serotype 1, 2, 3, or 4). A total of 25 field isolates and 10 laboratory strains examined by this method exhibited marked sequence identity in both areas of the gene with the corresponding regions of 1 of the 4 reference strains. In addition, the predicted serotype from the sequence analysis correlated in each case with the serotype determined when the rotaviruses were examined by plaque reduction neutralization or reactivity with serotype-specific monoclonal antibodies. These data suggest that as a result of the high degree of sequence conservation observed among rotaviruses of the same serotype, it is possible to predict the serotype of a rotavirus isolate by direct sequence analysis of its VP7 gene.

Four serotypes were originally identified among the many rotaviruses recovered from humans (25), and two new human rotavirus serotype candidates have been described recently (1, 5, 18). Rotavirus serotypes have been defined using plaque reduction neutralization (PRN) in vitro (16, 24), tube neutralization (25), fluorescent focus neutralization (3) and, recently, enzyme-linked immunosorbent assay (ELISA) using serotype-specific monoclonal antibodies (7, 15, 21, 22). The major serotype-specific neutralization antigens have been demonstrated (13, 17) to be located on the VP7 outer capsid protein which is encoded by gene 8 or 9. In a recent report from this laboratory, the close relatedness of the gene sequences encoding the VP7 of rotavirus strains belonging to the same serotype was demonstrated by RNA-RNA hybridization experiments (19). In these experiments, denatured double-stranded RNAs from field strains were hybridized to ^{32}P-labeled single-stranded RNAs (ssRNAs) transcribed in vitro from single-gene substitution rotavirus reassortants (in which the VP7 gene was derived from human strains belonging to each of the four epidemiologically important rotavirus serotypes), which allowed the deduction of the rotavirus serotype specificity. More recently, we reported that at least six specific regions of the VP7 protein that are divergent in amino acid sequence among different serotypes are highly conserved among rotaviruses of the same serotype (12). On the basis of these observations, we proposed that it may be possible to predict the serotype of a rotavirus field isolate by comparison of its VP7 gene nucleotide sequence with that of a reference virus from each serotype (serotypes 1 through 4).

In the current study, a method was developed for obtaining and sequencing ssRNA transcripts from rotaviruses partially purified either directly from stool material or after passage in tissue culture. The places and dates of collection of 25 human rotavirus field isolates and laboratory strains used in this study are shown in Table 1. Strains B, G, and C were obtained from I. Sarov (Ben Gurion University of the Negev, Beer-Sheva, Israel), and strains 3819, 5083, 2743, and 2899 were obtained from T. Naguib (Egyptian Organization for Biological Products and Vaccines, Agouza-Guiza, Egypt). Strain P118 was obtained from R. Dolin (University of Rochester, Rochester, New York), strain McN13 was obtained from R. Bishop (Royal Children's Hospital, Melbourne, Australia), and strains Hochi and Hosokawa were obtained from Y. Inaba (National Institute of Animal Health, Ibaraki, Japan). Stool specimens positive for rotavirus by the ELISA were made into a 10% suspension in phosphate-buffered saline. To grow virus in tissue culture, portions of some stool suspensions were treated with trypsin (5 μg/ml) for 30 to 60 min at 37°C before absorption onto MA-104 cell monolayers. Viruses were partially purified either from stool suspensions (2 ml) or from tissue culture harvests by fluorocarbon extraction, followed by centrifugation through a 30% sucrose cushion as described previously (9). ssRNAs were produced by transcription from partially purified virus, extracted once with phenol-chloroform, and precipitated with 3 volumes of ethanol. Additional purification of virus was not required to obtain sufficient ssRNAs for sequence analysis. Dideoxynucleotide sequencing of rotavirus RNA was performed as described previously (12) with two synthetic oligonucleotides, 5'CCATTGGATTACACAACCAT TC3' and 5'GCTACGTTTTCTCTTGGTCC3', targeted to nucleotides 531 through 552 and 801 through 819 of the transcript RNA encoding VP7, respectively. These primers were deduced from published sequences of the VP7 gene of

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TABLE 1. Comparison of the serotype designation of 35 human rotavirus strains as determined by three methods

Strain	Country of origin	Year collected	Serotype determined by following method:		
			PRN	MAb[a]	Sequence[b]
D	United States	1974	1	1	1
HN289	Venezuela	1981		1	1
HN256	Venezuela	1981		1	1
HN307	Venezuela	1981		1	1
M37	Venezuela	1982	1	1	1
B	Israel	1983		1	1
G	Israel	1983		1	1
P118	United States	1985		1	1
DS-1	United States	1976	2	2	2
HN126	Venezuela	1981	2	2	2
2743	Egypt	1982		2	2
2899	Egypt	1982		2	2
C	Israel	1983		2	2
V197	Venezuela	1985		2	2
V206	Venezuela	1986		2	2
P	United States	1974	3	3	3
McN13	Australia	1980	3	3	3
HN330	Venezuela	1981		3	3
HN257	Venezuela	1981		3	3
V251	Venezuela	1985		3	3
V460	Venezuela	1986		3	3
V101	Venezuela	1986		3	3
V52	Venezuela	1986		3	3
V96	Venezuela	1986		3	3
ST3	England	1975	4	4	4
HN11	Venezuela	1980		4	4
HN14	Venezuela	1980		4	4
HN5	Venezuela	1980		4	4
HN19	Venezuela	1980		4	4
VA70	Italy	1981	4	4	4
HN98	Venezuela	1981		4	4
3819	Egypt	1983		4	4
5083	Egypt	1983		4	4
Hosokawa	Japan	1983	4	4	4
Hochi	Japan	1983	4	4	4

[a] Monoclonal antibody ELISA performed as described by Taniguchi et al. (22).
[b] Nucleotide sequence analysis.

bovine rotavirus strain Nebraska calf diarrhea virus (11) and are complementary to two regions in the VP7 gene which appear to be conserved in all rotaviruses examined thus far. These two priming sites are each downstream from a region of the gene that codes for a discrete cluster of amino acids in VP7 which are conserved within a serotype but are divergent among serotypes. The two regions sequenced, nucleotides 307 through 351 and 670 through 711, correspond to amino acids 87 through 101 and 208 through 221, respectively.

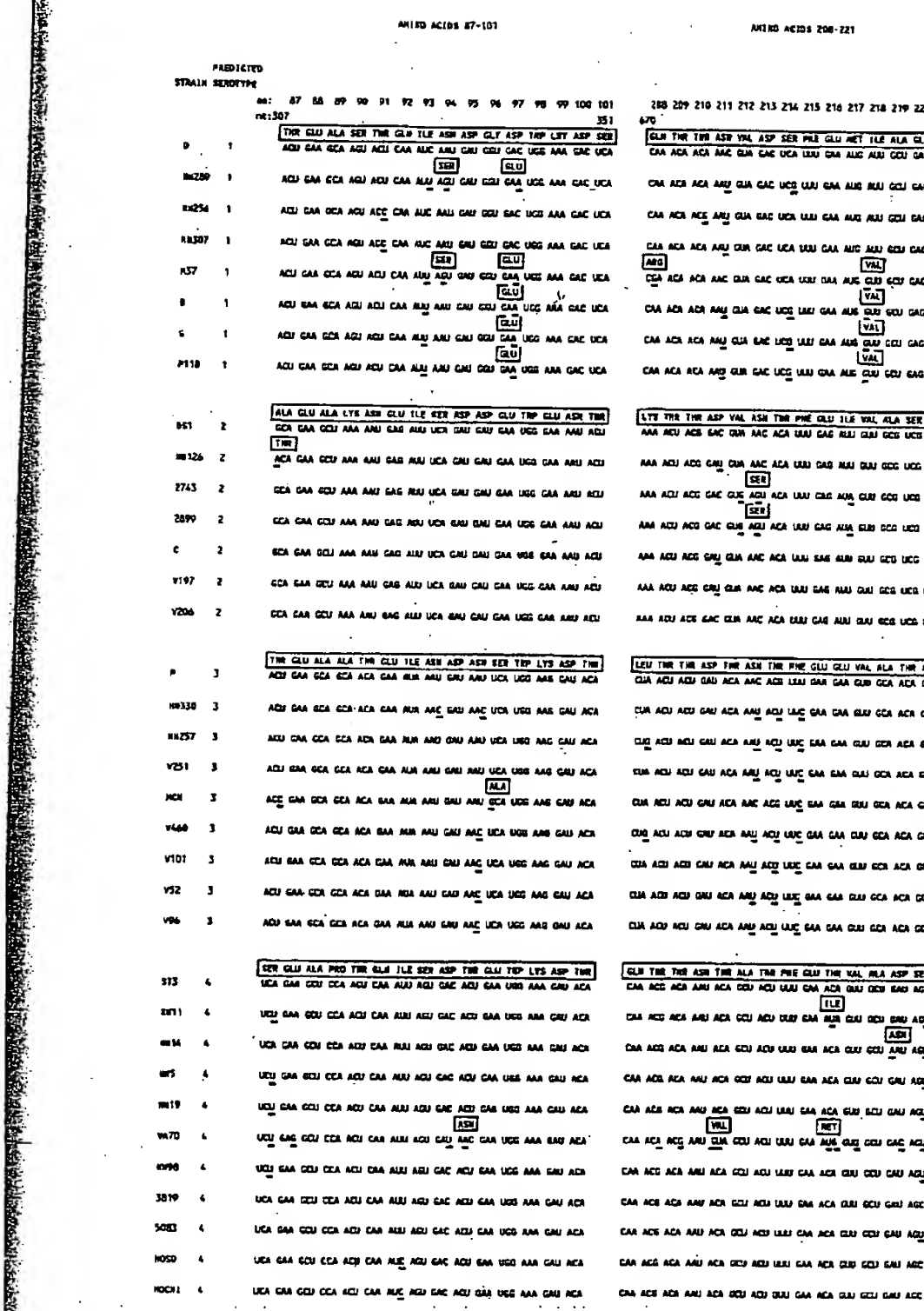
The nucleotide and deduced amino acid sequences obtained for each field strain in the two divergent regions were compared with the sequences of reference strains D (serotype 1), DS-1 (serotype 2), P (serotype 3), and ST3 (serotype 4). In Fig. 1, the sequence of each field strain is shown in comparison with the reference strain to which it showed the greatest identity. When there was marked conservation of amino acid sequence (≥85%) between a field strain and the reference strain in these two regions, the field strain was assigned the serotype of the reference strain. In contrast, amino acid similarity among different serotypes in these two regions ranged from 33 to 62%. No more than two amino acid substitutions occurred per region among strains of the same predicted serotype.

All 35 strains in this study were tested in a monoclonal antibody serotyping ELISA, and 10 were serotyped by PRN. A comparison of these results with the deduced serotype of each virus determined by sequence analysis showed complete agreement (Table 1).

Early after the discovery of rotaviruses in 1973, the adaptation of a series of animal and human strains to tissue culture and their comparison by neutralization assays permitted the designation of several serotypes (23). However, serotype identification has been difficult until recently. Serotype analysis of rotaviruses will undoubtedly be an essential component in the evaluation of rotavirus vaccine efficacy, because recent studies indicate the importance of serotype-specific immunity in humans. For example, phase II vaccine trials with a monovalent (serotype 3) vaccine, containing the rhesus rotavirus strain, have been conducted in several areas of the world, and the preliminary data indicate that the vaccine was successful in inducing significant resistance to severe diarrhea in an area where the prevalent serotype was the same as that of the vaccine strain (10) but was not successful in places where other serotypes were prevalent (17a).

Two regions of the VP7 gene shown to be conserved within a serotype but divergent among serotypes were sequenced from ssRNA transcripts of 25 rotavirus strains. These strains had been collected in widely scattered localities over a 12-year period. In every case, it was possible to correctly predict the serotype by comparing the sequence in two selected regions of the VP7 gene to those of strains of known serotype. These two regions of the gene were analyzed because they contained the sequences for amino acids (residues 94, 96, and 211) which had been previously assigned to two VP7 serotype-specific neutralization epitopes on the basis of sequence analysis of neutralization-resistant mutants selected with neutralizing monoclonal antibodies (8). Comparison of the nucleotide sequence of the isolates with the reference sequence to which it matched most closely showed that nucleotide substitutions did occur but that most substitutions were in position 3 of the codon and did not result in an amino acid substitution. Some nucleotide substitutions which resulted in an amino acid substitution

FIG. 1. Sequence analyses of 25 rotavirus field isolates of unknown serotype and 10 laboratory strains of known serotype. Nucleotide sequences from two areas of the VP7 gene transcript RNA (nucleotides 307 through 351 encoding amino acids 87 through 101 and nucleotides 670 through 711 encoding amino acids 208 through 221) which were divergent in sequence among serotypes were determined and compared with reference strains D (serotype 1), DS-1 (DS1) (serotype 2), P (serotype 3), or ST3 (serotype 4). The deduced amino acid sequences for each reference strain in both regions are enclosed in open boxes above the nucleotide sequence. The nucleotide sequences determined from each strain analyzed are shown beneath the reference strain to which the highest degree of similarity was detected. Nucleotide substitutions within strains corresponding to the same serotype are underlined, and amino acid substitutions are enclosed in open boxes. Each strain analyzed matched closely with only one of the reference strains in both divergent regions. The serotype of the reference strain to which the field isolate correlated was assigned as the predicted serotype of the field isolate. nt, Nucleotide; aa, amino acid.


AMINO ACIDS 87-101
AMINO ACIDS 208-221
PREDICTED
STRAIN SEROTYPE
aa: 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101
nt:307 351
208 209 210 211 212 213 214 215 216 217 218 219 220 221
670 711
THR GLU ALA SER THR GLN ILE ASN ASP GLY ASP TRP LYS ASP SER
GLN THR THR ASP VAL ASP SER PHE GLU MET ILE ALA GLU ASN
D 1
HN289 1
HN256 1
HN307 1
N37 1
B 1
G 1
P118 1
ALA GLU ALA LYS ASN GLU ILE SER ASP ASP GLU TRP GLU ASN THR
LYS THR THR ASP VAL ASN THR PHE GLU ILE VAL ALA SER SER
B61 2
HN326 2
2743 2
2899 2
C 2
V197 2
Y206 2
THR GLU ALA ALA THR GLU ILE ASN ASP ASN SER TRP LYS ASP THR
LEU THR THR ASP THR ASN THR PHE GLU GLU VAL ALA THR ALA
P 3
HN338 3
HN257 3
V251 3
HCH 3
V460 3
V101 3
V52 3
V96 3
SER GLU ALA PRO THR GLN ILE SER ASP THR GLU TRP LYS ASP THR
GLN THR THR ASN THR ALA THR PHE GLU THR VAL ALA ASP SER
S73 4
HN11 4
HN14 4
HN5 4
HN19 4
VA70 4
HN98 4
3819 4
5083 4
NOSO 4
HOCH1 4

were located at positions to which amino acid substitutions in monoclonal antibody-selected variants had been mapped. In addition, sequence analysis was performed in these two divergent regions of the gene approximately 400 bases apart to determine whether field isolates maintained serotype-specific sequences throughout the gene or whether recombination of divergent regions occurred. Evidence for chimeric VP7 sequences was not obtained. Each of the 25 isolates possessed both of the variable region sequences of the reference rotavirus it most closely resembled. In addition, the isolates matched closely with the reference virus in other regions of the VP7 gene (including nucleotides 163 through 198 and 745 through 774) shown to be variable among different serotypes (data not shown). However, since the neutralization specificity of VP7 appears to be dependent on protein conformation (2, 14), it is possible that strains may exist which contain mixed divergent regions which yield a new serotype specificity.

Taniguchi et al. (22), using monoclonal antibodies specific for the VP7 of rotaviruses from serotypes 1 through 4, identified the serotype of rotaviruses in 38 out of 57 rotavirus-positive stool specimens examined. Of the 19 specimens studied that were not typed, 10 of these appeared to contain two viruses, 4 were nonreactive with the VP3 common antigen control antibody, and 5 reacted with the VP3 common antigen control antibody but did not react with any of the serotype-specific monoclonal antibodies. These latter viruses could represent either new serotypes or naturally occurring variants. Coulson (6) has described the existence of serotype 1 rotaviruses with various reactivity patterns with three different serotype 1-specific monoclonal antibodies and has proposed that these variants be designated monotypes. It is apparent from sequence data presented here and previously that although there is a high degree of sequence conservation within rotaviruses of the same serotype, there are sites in the VP7 gene where genetic variation occurs. The emergence of variants from the genetic pool of rotaviruses could explain the different reactivities of some rotaviruses with certain monoclonal antibodies, i.e., monotypes. It is not known whether existing host antibody pressures play a role in the emergence of these variants. However, it is evident that care must be used in the selection of monoclonal antibodies for a serotyping assay. For example, serotype 1-specific monoclonal antibody 2C9, described by Shaw et al. (21), neutralizes serotype 1 strain D but does not neutralize serotype 1 strain M37 by PRN. Strain M37 contains an amino acid substitution (Asn → Ser) at residue 94 when compared with strain D and thus could represent a naturally occurring variant which would not be recognized by monoclonal antibody 2C9, which maps to residue 94.

The serotype of each test strain predicted by the sequence analysis in this study correlated in each case with the serotype of the strain determined by a monoclonal antibody serotyping ELISA. Furthermore, PRN assays performed with a number of these specimens also yielded results indicating concordance with sequence analyses. Although sequence analysis may not be a practical method for routine typing in most laboratories, sequencing of VP7 genes has several features of practical interest. (i) If sufficient virus is present in the stool, rotavirus particles can be partially purified and transcribed from the stools without adaptation of the virus to growth in tissue culture and, if not, two passages in tissue culture will provide sufficient virus. (ii) Comparison with the reference strains will reveal at the molecular level whether the strain is a variant or a new serotype. Sequence analysis has been used recently in another virus system for a similar purpose. Subgrouping of foot-and-mouth disease virus by sequence analysis revealed that several outbreaks of the disease in Europe were likely caused by a subtype of the virus identical to the vaccine strain (4). Genotyping of poliovirus is now being used for epidemiologic studies (20). In the latter case, direct sequence analysis was performed on an area of the genome relevant to serotype. Similar studies with rotaviruses are in progress in our laboratory using sequence analysis as an epidemiologic tool.

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Characterization of the Neutralizing Epitopes of VP7 of the Gottfried Strain of Porcine Rotavirus†

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The neutralization epitopes of the outer capsid protein VP7 of a porcine group A rotavirus were studied by using neutralizing monoclonal antibodies (N-MAbs). Six N-MAbs which were specific for the VP7 protein of the Gottfried strain of porcine rotavirus (serotype G4) were used for analyzing the antigenic sites of VP7. Three different approaches were used for this analysis: testing the serological reactivity of each N-MAb against different G serotypes of human and animal rotaviruses, analyzing N-MAb-resistant viral antigenic variants, and performing a nucleotide sequence analysis of the VP7 gene of each of the viral antigenic variants generated. From the serological analyses, three different reactivity patterns were recognized by plaque reduction virus neutralization and cell culture immunofluorescence tests. A single MAb (RG36H9) reacted with animal rotavirus serotypes G3 and G4 but not with human serotypes G3 and G4. The MAb 57/8 (D. A. Benfield, E. A. Nelson, and Y. Hoshino, p. 111, *in* Abstr. VIIth Internat. Congr. Virol., 1987, and E. R. Mackow, R. D. Shaw, S. M. Matsui, P. T. Vo, D. A. Benfield, and H. B. Greenberg, Virology 165:511–517, 1988) reacted with animal and human rotavirus serotypes G3 and G4 and also with human serotype G9 and bovine serotype G6. The other four MAbs reacted only with the porcine rotavirus serotype G4. The epitope defined by MAb 57/8 and the epitope defined by the other five MAbs appeared to be partially overlapping or close to each other, as identified by viral antigenic variant analysis. However, data from nucleotide and deduced amino acid sequence analyses of the VP7 of each of the viral antigenic variants showed that these two epitopes constituted a large, single neutralization domain.

Rotaviruses are established causes of acute gastroenteritis in humans, as well as in other mammals and in avian species (9, 21). At least four G serotypes of group A rotavirus bearing VP7 have been identified among strains isolated from pigs (3, 9, 20). Of the porcine rotaviruses, the Gottfried strain is antigenically related to serotype G4 human rotaviruses (3, 20) whereas the serotype G5 OSU strain is antigenically distinct from known human rotavirus serotypes (3). Other porcine rotavirus isolates have been classified as serotype G3, serotype G11, and mixed serotypes of G3 and G5 (28, 31). Recently, new candidate porcine rotavirus serotypes have been described but not fully characterized (1, 30).

Two outer capsid proteins of group A rotaviruses, VP4 and VP7, are now known to be independent neutralization antigens (15, 16, 18, 19). It has been shown that the serotype-specific antigen involved in virus neutralization is mainly defined by the VP7 glycoprotein (serotype G), which is encoded by RNA segment 7, 8, or 9 (15, 16). By utilizing comparative analysis of nucleotide and deduced amino acid sequences of VP7 genes from different animal and human rotavirus serotypes, nine discrete regions of divergence (VR1 to VR9) were defined (13). Six of these regions were previously defined as divergent regions among serotypes (12, 14, 17).

In the present study, we produced VP7-specific neutralizing monoclonal antibodies (N-MAbs) against the Gottfried strain of porcine rotavirus and used them to characterize the neutralizing epitopes of VP7. The reactivities of these N-MAbs with distinct serotypes of animal rotaviruses and symptomatic and asymptomatic human rotaviruses were examined by plaque reduction virus neutralization (PRVN) and cell culture immunofluorescence (CCIF) tests. Viral antigenic variants resistant to the VP7-specific N-MAbs were selected for and analyzed. In addition, the nucleotide changes and the deduced amino acid changes within VP7 associated with the antigenic alteration in the variants were examined.

MATERIALS AND METHODS

Viruses and cells. The following pairs of symptomatic and asymptomatic human rotaviruses, each pair serologically distinct, were used for characterizing the N-MAbs: Wa and M37 (serotype G1), DS-1 and 1076 (serotype G2), M and McN13 (serotype G3), and VA70 and ST-3 (serotype G4). These human rotavirus strains were provided by Y. Hoshino (National Institutes of Health, Bethesda, Md.). Human rotavirus strain WI61 (serotype G9) (6), supplied by H. F. Clark (The Wistar Institute, Philadelphia, Pa.), was included as a symptomatic human rotavirus. The following were included as animal rotaviruses: simian rotaviruses SA-11 and RRV (serotype G3), obtained from M. K. Estes (Baylor College of Medicine, Houston, Tex.) and Y. Hoshino, respectively, the Gottfried strain of porcine rotavirus (serotype G4); porcine rotavirus strains OSU (serotype G5) and SB-1A (with a serotype G4 VP7 and VP4 like that of OSU); and bovine rotaviruses NCDV Lincoln (serotype G6) and

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B223 (serotype G10, supplied by G. N. Woode [Texas A&M University, College Station, Tex.]). Other serotype G5 porcine rotaviruses (EE and A580) (3, 20), isolated and passaged in our laboratory, were also used. All rotaviruses were plaque purified three times before use.

An African green monkey kidney cell line (MA104) was used throughout this study. The cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U of penicillin, 100 μg of streptomycin, and 25 μg of nystatin per ml).

Production of MAbs. Hybridomas were produced by procedures similar to those used previously in our laboratory (22, 35). Female BALB/c mice, 8 to 10 weeks old, were maintained in plastic isolators under gnotobiotic isolation conditions. Mice maintained in a similar manner were also used for ascites production. The mice were immunized intraperitoneally by using complete Freund's adjuvant with the Gottfried strain of porcine rotavirus, semipurified from the gut contents of an infected gnotobiotic pig. Two weeks later, virus was administered intraperitoneally in incomplete Freund's adjuvant. After an additional 2 weeks, mice were given intravenous boosters of virus in phosphate-buffered saline (PBS) (pH 7.4). Three days after the final booster, spleen cells were fused with SP2/0 myeloma cells by using 50% polyethylene glycol (molecular weight, 3,350; Sigma Chemical Co., St. Louis, Mo.). When hybridomas occupied at least 30% of the well area, culture supernatants were screened for production of neutralizing antibody against the Gottfried strain of porcine rotavirus by a fluorescent focus neutralization (FFN) test. Hybridomas which neutralized the Gottfried strain of porcine rotavirus during the FFN test were cloned at least twice by limiting dilution with mouse thymocyte feeder layers. The immunoglobulin isotype of each MAb was determined by using hybridoma culture fluids in Ouchterlony double immunodiffusion gels with sheep anti-mouse immunoglobulin typing sets (ICN ImmunoBiologicals, Lisle, Ill.).

Pristane-primed BALB/c mice were injected intraperitoneally with cloned hybridoma cells to produce ascitic fluids. The ascitic fluids were filtered (0.45-μm-pore-size filter; Millipore Corp., Bedford, Mass.), heat inactivated (56°C for 30 min), and stored frozen until used in the assays.

FFN test. The FFN test was performed to screen for hybridomas secreting neutralizing antibodies against the Gottfried strain of porcine rotavirus by a modification of methods previously described (22, 35). Briefly, equal volumes of hybridoma culture fluids and virus suspensions containing 10^4 fluorescent focus-forming units per 0.50 ml were mixed and incubated for 1 h at 37°C. MA104 cell monolayers in 96-well plates were inoculated with these mixtures and incubated at 37°C for 18 to 20 h in a 5% CO_2 atmosphere and then fixed with 80% acetone and stained with fluorescein isothiocyanate-conjugated porcine anti-porcine rotavirus (OSU strain) immunoglobulin. Hybridoma supernatants that reduced fluorescent focus-forming units by 60% or more were considered to contain neutralizing antibodies to the Gottfried strain of porcine rotavirus.

CCIF test. The CCIF test was performed to determine the reactivity patterns of N-MAbs against different serotypes of human and animal rotaviruses. Confluent MA104 cell monolayers in 96-well microtiter plates were infected with various human and animal rotaviruses at a multiplicity of infection of 0.1 PFU per cell. After incubation at 37°C for 18 to 20 h, infected cells were rinsed once with PBS and fixed with 80% acetone. Immunoglobulins precipitated from the ascitic fluids with ammonium sulfate and mixed with PBS at a standard concentration of 10 μg/ml were added to infected, fixed cells and incubated for 1 h at 37°C. After incubation, the plates were washed three times with PBS and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (A, G, and M) (Kirkegaard and Perry, Gaithersburg, Md.) were added to each well at a 1:30 dilution. Following 1 h of incubation at 37°C, the plates were washed twice with PBS. Cells were examined for immunofluorescence with a fluorescence microscope (Olympus IM, Tokyo, Japan), and wells which contained cells fluorescing in the presence of N-MAbs were considered positive. For determination of the titers of the N-MAbs against the homologous Gottfried strain of porcine rotavirus by the CCIF test, serial dilutions of unconcentrated ascites were tested as described above. The titer was expressed as the reciprocal of the highest ascites dilution which resulted in the appearance of fluorescing cells.

PRVN test. The PRVN test was performed in plastic six-well plates containing MA104 cell monolayers to determine titers of the N-MAbs as previously described (35). In brief, serial fourfold dilutions of ascitic fluids were mixed with equal volumes of the viral suspension, containing 60 to 80 PFU/0.1 ml, and incubated at 37°C for 1 h. Then, 0.1-ml volumes of the mixtures were inoculated onto duplicate wells and incubated for an additional 1 h at 37°C. The plates were washed once with serum-free EMEM, and agar medium containing 0.8% noble agar and 67 μg of neutral red and 25 μg of pancreatin (GIBCO Laboratories, Grand Island, N.Y.) per ml (four times the neutralization factor) in EMEM was added. The PRVN titer was expressed as the reciprocal of the highest dilution of ascitic fluid which produced an 80% reduction in the number of plaques compared with the number in wells containing the control virus.

Production of N-MAb-resistant viral antigenic variants. The VP7-specific N-MAbs were used to select viral antigenic variants which were resistant to neutralization. Selection and isolation of the viral antigenic variants were performed by a modification of procedures previously described (33). In brief, 100 μl of serial 10-fold dilutions of the Gottfried strain of porcine rotavirus was mixed with an equal volume of ascitic fluids diluted 1:25 to 1:500 in EMEM and incubated at 37°C for 1 h. MA104 cell monolayers grown in roller tubes were then inoculated with this mixture. After adsorption for 1 h, cells were washed with serum-free EMEM and fed with 2.0 ml of serum-free EMEM containing ascitic fluid diluted 1:500 or 1:2,000 and pancreatin. Infected cells were incubated in a roller tube apparatus and were harvested after 7 days. The growth of each viral antigenic variant was assayed by a CCIF test, and the resistance of each variant to the corresponding N-MAb was confirmed by the FFN test. After an additional two cycles of virus neutralization and multiplication, as described above, the resultant viral antigenic variants were plaque purified at least twice in MA104 cells. Each viral antigenic variant was designated by prefixing the name of the N-MAb used for its selection with the letter v. We picked five plaques for each selecting MAb, and the plaque-purified viral antigenic variants were examined for their patterns of reactivity with the panel of VP7-specific N-MAbs.

Protein specificity of the N-MAbs. The protein specificity of each N-MAb was determined by the PRVN test with the naturally occurring reassortant porcine rotavirus strain SB-1A. The SB-1A strain contains all the protein genes of the Gottfried strain (including the VP7 gene, serotype G4) except for the VP4 gene (serotype P), which is of OSU origin (3, 18, 20).

Nucleotide sequences of viral antigenic variants. The nucle-

TABLE 1. Characterization by isotype, protein specificity, and titer of MAbs produced against the Gottfried strain of porcine rotavirus

MAb[a]	Immunoglobulin isotype	Antibody titer[b] in test	
		PRVN	CCIF
RG36H9	M	270,000	102,400
RG38A3	M	180,000	12,800
RG39C12	G3	64	<100
RG42A6	G3	64	<100
RG43H1	G3	64	<100
57/8	M	350,000	>204,800

[a] The protein specificity of the MAbs (from ascites) was VP7, as determined by reaction with an OSU × Gottfried reassortant rotavirus (SB-1A) (VP4: OSU; VP7:Gottfried).

[b] PRVN titers against the Gottfried strain of rotavirus are expressed as the reciprocal of the ascites dilution which produced an 80% reduction in plaques compared with the number of plaques in controls. Titers in the CCIF test represent the reciprocal of the ascites dilution which produced immunofluorescence after indirect staining of cell cultures infected with the Gottfried strain of rotavirus with fluorescein-conjugated anti-mouse immunoglobulins.

otide sequences of the VP7 gene of the Gottfried strain of porcine rotavirus and the viral antigenic variants were determined by primer extension analysis (14). Plus-stranded RNA transcripts were prepared from single-shelled rotavirus cores, and dideoxynucleotide sequencing of RNA transcripts was performed by a method described previously (10).

RESULTS

Characterization of MAbs. Both VP4- and VP7-specific N-MAbs were produced during cell fusion, and VP4-specific N-MAbs were characterized previously (22). A panel of six VP7-specific N-MAbs was used in this study, five of which were produced and cloned in our laboratory. The sixth N-MAb, 57/8, was produced previously by Benfield et al. (2). A summary of the properties of these N-MAbs is given in Table 1. All five N-MAbs were shown to be VP7 specific by their neutralization of the Gottfried and SB-1A rotavirus strains (Table 2). The latter virus shares the VP7 G4 serotype with the Gottfried rotavirus strain but contains an unrelated gene 4 (a VP4 gene like that of OSU rotavirus). A Gottfried VP4-specific N-MAb, RG24B9 (22), was included in the assays as a control. This N-MAb (RG24B9) failed to neutralize rotavirus strain SB-1A.

Reactivity of N-MAbs with various rotavirus strains. (i) **Virus neutralization.** The cross-reactivity of the N-MAbs specific for VP7 of the Gottfried strain with various serotypes of human and animal rotaviruses, determined by the PRVN test, is shown in Table 2. Three distinct patterns of reactivity of the N-MAbs with various human and animal rotavirus serotypes were observed. On the basis of the PRVN results, VP7-specific N-MAbs were divided into three groups (groups I to III) according to their patterns of reactivity with various serotypes of human rotavirus. One N-MAb, categorized as group I (RG36H9), did not neutralize any of the human rotavirus serotypes tested, whereas it reacted with animal rotaviruses of the G3 serotype (SA-11 and RRV). However, another four N-MAbs (RG38A3, RG39C12, RG42A6, and RG43H1), categorized as group II, did not neutralize any of the human and animal rotavirus serotypes examined except for the Gottfried and SB-1A strains (serotype G4). The single N-MAb in group III, 57/8, cross-reacted with symptomatic and asymptomatic human rotavirus serotypes G3 and G4 and with symptomatic human rotavirus serotype G9 (Table 2). This N-MAb also neutralized animal rotavirus serotypes G3 (SA-11 and RRV) and G6 (NCDV). As indicated earlier, all the VP7-specific N-MAbs reacted with SB-1A porcine rotavirus, which shares the VP7 gene with the Gottfried strain of rotavirus and the VP4 gene with the OSU strain of rotavirus (11).

(ii) **CCIF.** The reactivity patterns of the N-MAbs obtained with various serotypes of human and animal rotaviruses, as determined by a CCIF test, are shown in Table 3. On the basis of these results, the N-MAbs were grouped (groups I, II, and III) as described above.

Viral antigenic variant analysis. To map the topographical antigenic sites of VP7, we selected viral antigenic variants from a pool of the Gottfried strain grown in MA104 cells in the presence of each of the N-MAbs. We did not calculate the frequency of viral antigenic variants. However, viral antigenic variants could be readily isolated by three passages

TABLE 2. PRVN titers[a] of Gottfried VP-7-specific MAbs against homologous virus, symptomatic and asymptomatic human rotavirus G serotypes, and animal rotavirus G serotypes

Group and MAb (from ascites)	Titer(s) against rotavirus (serotype)[b]										
	Human strain(s)					Animal strain(s)					
	Wa (G1)/ M37 (G1)	DS-1 (G2)/ 1076 (G2)	M (G3)/ McN13 (G3)	VA70 (G4)/ ST-3 (G4)	WI61 (G9)	SA-11 (G3)	RRV (G3)	Gottfried (G4)/ SB-1A (G4)	OSU (G5)	NCDV (G6)	B223 (G10)
I											
RG36H9	<4/<4	<4/<4	<4/<4	<4/<4	<4	3,600	7,000	270,000/270,000	<4	<4	<4
RG38A3	<4/<4	<4/<4	<4/<4	<4/<4	<4	<4	<4	180,000/100,000	<4	<4	<4
II											
RG39C12	<4/<4	<4/<4	<4/4	<4/<4	<4	<4	<4	64/64	<4	<4	<4
RG42A6	<4/<4	<4/<4	<4/4	<4/<4	<4	<4	<4	64/64	<4	<4	<4
RG43H1	<4/<4	<4/<4	<4/4	<4/<4	<4	<4	<4	64/64	<4	<4	<4
III											
57/8	<4/<4	<4/<4	>65,536/300	>65,536/9,300	4,096	>65,536	>65,536	350,000/>65,536	<4	20,000	<4

[a] Expressed as the reciprocal of the ascites dilution which produced an 80% reduction in plaques.

[b] Human strains are in symptomatic/asymptomatic pairs; animal strains (symptomatic) are paired by serotype only. The human strain listed singly is symptomatic.

TABLE 3. Patterns of reactivity of Gottfried VP7-specific MAbs against various human and animal rotavirus G serotypes in the CCIF test[a]

Group and MAb	Pattern of reactivity with rotavirus (serotype)[b]									
	Human strain(s)					Animal strain(s)				
	Wa (G1)/ M37 (G1)	DS-1 (G2)/ 1076 (G2)	M (G3)/ McN13 (G3)	VA70 (G4)/ ST-3 (G4)	WI61 (G9)	SA-11 (G3)/ RRV (G3)	Gottfried (G4)/ SB-1A (G4)	SB-2 (G4)/ SB-5 (G4)	OSU (G5)/ EE (G5)	NCDV (G6)/ B223 (G10)
I										
RG36H9	−/−	−/−	−/−	−/−	−	+/+	+/+	−/−	−/−	−/−
II										
RG38A3	−/−	−/−	−/−	−/−	−	−/−	+/+	−/−	−/−	−/−
RG39C12	−/−	−/−	−/−	−/−	−	−/−	+/+	−/−	−/−	−/−
RG42A6	−/−	−/−	−/−	−/−	−	−/−	+/+	−/−	−/−	−/−
RG43H1	−/−	−/−	−/−	−/−	−	−/−	+/+	−/−	−/−	−/−
III										
57/8	−/−	−/−	+/+	+/+	+	+/+	+/+	−/−	−/−	+/−
RG25A10[c]	+/+	+/+	+/+	+/+	+	+/+	+/+	+/+	+/+	+/+

[a] Ammonium sulfate-precipitated MAbs from ascites were tested at a concentration of 10 μg/ml against MA104 cells infected with various rotaviruses, incubated in 96-well tissue culture plates. +, presence of specific fluorescing cells; −, absence of specific fluorescing cells.

[b] Human strains are in symptomatic/asymptomatic pairs; animal strains (symptomatic) are paired by serotype only. The human strain listed singly is symptomatic.

[c] VP6-specific MAb used as a control.

of the virus in the presence of the N-MAb in roller tube cell cultures prior to plaque purification (data not shown).

We attempted to isolate different kinds of viral antigenic variants against each N-MAb, on the basis of distinctive plaque characteristics (size, opacity, etc.) which might cause different neutralization reactivity patterns with the panel of VP7-specific N-MAbs. We obtained eight viral antigenic variants by using N-MAbs directed against the Gottfried strain of porcine rotavirus. The patterns of reactivity between these eight viral antigenic variants and the panel of VP7-specific N-MAbs, determined by cross-neutralization tests, are summarized in Fig. 1. Three distinct reactivity patterns were observed. RG36H9, categorized as group I, selected one viral antigenic variant, v-RG36H9, which was resistant to neutralization not only by the homologous N-MAb used for its selection but also by four heterologous N-MAbs (RG38A3, RG39C12, RG42A6, and RG43H1). However, v-RG36H9 was sensitive to neutralization by N-MAb 57/8. Two N-MAbs (RG42A6 and G43H1) categorized as group II each selected two distinct viral antigenic variants, v-RG42A6/A and v-RG42A6/B for RG42A6 and v-RG43H1/A and v-RG43H11/B for G43H1. They behaved differently with the panel of VP7-specific N-MAbs. For example, v-RG42A6/A was sensitive to N-MAb RG38A3, whereas v-RG42A6/B was resistant to neutralization by the same N-MAb. However, two N-MAbs (RG39C12 and RG38A3), also categorized as group II, selected viral antigenic variants v-RG39C12 and v-RG38A3, respectively, and their reactivity patterns were the same as that of v-RG36H9. The group III N-MAb, 57/8, selected one viral antigenic variant, and its reactivity pattern was the same as that of v-RG43H1/A. Only v-RG43H1/A and the homologous variant (v-57/8) were resistant to neutralization by N-MAb 57/8. v-RG42A6/A was resistant to neutralization by the homologous N-MAb RG42A6 and by heterologous N-MAbs RG36H9 and RG39C12, but it was sensitive to RG38A3.

Nucleotide sequence analysis of antigenic variants. To map the antigenic sites on VP7 of the Gottfried strain, we sequenced the VP7 gene of the parent Gottfried strain and that of its antigenic variants which were resistant to the N-MAbs used for their selection. Table 4 shows the nucleotide sequence of the VP7 gene and the deduced amino acid sequence changes in the viral antigenic variants. Only single nucleotide changes were found on the entire VP7 gene of each viral antigenic variant.

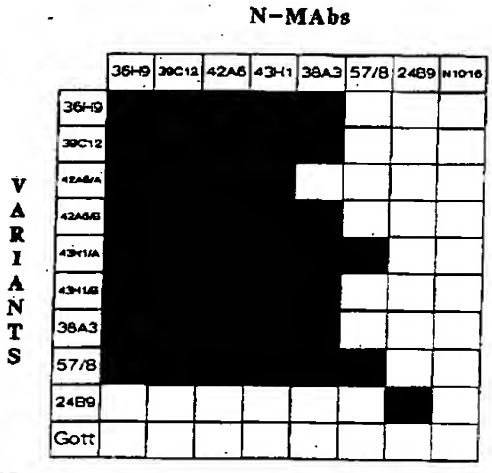

FIG. 1. Antigenic sites on VP7 of the Gottfried strain of porcine rotavirus. A panel of VP7-specific N-MAbs was tested for neutralization of the parent virus (Gott) and the N-MAb-resistant viral antigenic variants by the FFN test. The viral antigenic variants were designated as resistant (■) or sensitive (□) to neutralization with each N-MAb. The criterion for resistance was that the titer of the N-MAb against a given variant be at least 32-fold lower than the titer against the parent virus. Viral antigenic variant v-RG24B9, which is resistant to VP4-specific N-MAb RG24B9, was included as a control. N1016 represents porcine hyperimmune anti-Gottfried porcine rotavirus serum.

TABLE 4. Nucleotide and amino acid sequence changes found in the viral antigenic variants selected with VP7-specific N-MAbs

Antigenic variant	Selecting N-MAb	Codon change (position)	Amino acid change (position)
v-RG36H9	RG36H9	AAT to GAT (328)	Asn to Asp (94)
v-RG38A3	RG38A3	AAT to GAT (328)	Asn to Asp (94)
v-RG39C12	RG39C12	AAT to GAT (328)	Asn to Asp (94)
v-RG42A6/A	RG42A6	AAT to AGT (335)	Asn to Ser (96)
v-RG42A6/B	RG42A6	AAT to ACT (335)	Asn to Thr (96)
v-RG43H1/A	RG43H1	AAT to GAT (335)	Asn to Asp (96)
v-RG43H1/B	RG43H1	AAT to GAT (335)	Asn to Asp (96)
v-57/8	57/8	AAT to GAT (328)	Asn to Asp (94)

DISCUSSION

N-MAbs directed against the VP7 of strains SA-11 and RRV, three serotypes of porcine rotaviruses, and human rotavirus strains have been described previously (4, 7, 16, 27, 32, 34). We repeatedly failed to determine the viral protein specificities of our VP7-specific MAbs by Western blot (immunoblot) analysis (data not shown), confirming other reports that many VP7-specific N-MAbs do not react with denatured VP7 in a Western blot or by immunoprecipitation (7, 11, 34). However, each of our N-MAbs was confirmed as VP7 specific by its neutralization of the naturally occurring reassortant porcine rotavirus strain SB-1A, which contains an unrelated gene 4 (a VP4 gene) from a serotype G5 (OSU strain) rotavirus but gene 9 (a VP7 gene) from a serotype G4 (Gottfried strain) rotavirus (19).

Morita et al. (26) also determined the protein specificities of N-MAbs using gene reassortants. However, protein specificity determined only by using reassortant viruses should be analyzed carefully, on the basis of the results obtained by Chen et al. (5), who showed that VP4-specific MAbs could react in reassortant studies as if they recognized VP7 because of steric interactions between VP4 and VP7. In our experiment, all the N-MAbs tested neutralized the SB-1A reassortant rotavirus (Table 2). VP4-specific N-MAbs to the Gottfried strain of rotavirus were produced and characterized previously, and protein specificity was determined by an immunoprecipitation test. None of the VP4-specific N-MAbs neutralized SB-1A, further confirming their VP4 specificity (22).

N-MAbs and their viral antigenic variants are useful for analyzing the antigenic determinants of viral proteins which are involved in neutralization (8, 24–26, 32, 33). The genetic changes sustained by each viral antigenic variant which is resistant to the selecting N-MAb should also confer resistance to other N-MAbs which recognize similar antigenic determinants.

Although differences in the serological reactivity patterns suggested three distinct groups of N-MAbs, the viral antigenic variant analyses confirmed nonreciprocal reactions indirectly linking at least three neutralization epitopes within a single domain (Fig. 1). Competitive binding experiments with our N-MAbs will be required to elucidate the structural relationship between the identified epitopes. Viral antigenic variant analysis can create a relative map of the antigenic topography of the viral surface and can identify the amino acids that directly or indirectly affect neutralization epitopes, but it cannot identify the exact location of neutralization epitopes. When the nucleotide sequence of the VP7 gene of each viral antigenic variant was compared with that of the VP7 gene of the parent virus, only a single nucleotide change was found on the entire VP7 gene in each viral antigenic variant. All viral antigenic variants selected with N-MAbs had an amino acid change in region A (amino acids 87 to 101). The VP7-specific N-MAbs did not select any viral antigenic variants that had a mutation in region B (amino acids 145 to 150) or region C (amino acids 211 to 213). Except for this difference, our observations with VP7-specific N-MAbs against the Gottfried strain of porcine rotavirus (serotype G4) are similar to those previously reported for other rotavirus serotypes (25, 26, 32, 33).

On the basis of VP7 gene sequence analysis of neutralization-resistant antigenic variants selected with serotype G3-specific homotypic N-MAbs against SA-11 rotavirus, Dyall-Smith et al. (8) identified three distinct regions (A, B, and C) related to neutralization, two of which (A and C) appeared to be close together on the native VP7 glycoprotein. Also, the same large neutralization domain with several interrelated epitopes on VP7 was defined by several investigators using different serotypes of rotaviruses (26, 32, 33). These findings suggest that the immunodominant antigenic sites for neutralization on VP7 resides are at the same positions in rotaviruses which are of different serotypes as well as from different species.

Recently, however, Kobayashi et al. (23) identified a new neutralization epitope (S-2) on VP7, which is distinct from the previously recognized ones. There are several reports describing heterotypic epitopes on rotavirus VP7 that induce cross-neutralizing antibodies. Dyall-Smith et al. (8) reported that there is a close spatial relationship between the A and C regions; in their study, antigenic variants in both regions were selected with the same N-MAb. Mackow et al. (25) demonstrated that even though heterotypic N-MAb 57/8 binds to an antigenic site made up of both the A and C regions, the variants selected with this N-MAb had a mutation at amino acid 94 in the A region, the same amino acid location selected with homotypic N-MAbs. They proposed the hypothesis that VP7-specific heterotypic N-MAbs recognize epitopes composed of A and C regions which are conformation dependent. Taniguchi et al. (33) showed that one heterotypic N-MAb specific for VP7 of serotypes G1, G3, and G4 selected variants with mutations at the same position in region A (amino acid 96, 98, or 99). Our experiments showed similar results. Viral antigenic variants selected with the heterotypic N-MAbs RG36H9 (which neutralizes serotypes G3 and G4) and 57/8 (which neutralizes serotypes G3, G4, G6, and G9) had a mutation within the A region (amino acid 94, Asn → Asp), and serotype-specific neutralization epitopes recognized by N-MAbs RG38A3, RG39C12, RG42A6, and RG43H1 mapped in the same region (amino acid 94 or 96, Asn → Asp or Asn → Ser or Thr). Although the homologous PRVN titers of three of the group II MAbs (RG39C12, RG42A6, and RG43H1) were low (titers of 64), they were effective in inducing escape mutations in this known antigenic site and they displayed reactivity patterns similar to that of a fourth MAb in group II (RG38A3) with a high PRVN titer (180,000). Because we did not recover viral antigenic variants with mutations in region C (amino acids 211 to 213), we could not test and confirm the conclusions of Mackow et al. (25) regarding heterotypic neutralization epitopes composed of A and C regions. More VP7-specific N-MAbs are needed to resolve this question.

The role of VP7 in heterotypic immunity is unclear. At first, VP4 appeared to be responsible for the induction of heterotypic immunity (18, 19, 29). Several VP4-specific N-MAbs were cloned and shown to be cross-reactive with different serotypes of human and animal rotaviruses (22). However, we and others produced VP7-specific N-MAbs

that neutralized different serotypes of human and animal rotaviruses (2, 25, 33), suggesting that VP7 also plays a role in heterotypic immunity to rotaviruses.

In summary, we selected viral antigenic variants to be tested against a panel of VP7-specific N-MAbs, and three distinct patterns of reactivity between these variants and the N-MAbs were observed. The N-MAbs showed three different patterns of reactivity against various G serotypes of human and animal rotaviruses in PRVN and CCIF tests. Nevertheless, viral antigenic variants were selected at two closely spaced amino acids (Asn-94 and -96), resulting in a change to one of three other amino acids (Asp, Ser, or Thr). These results imply that the actual binding sites of the N-MAbs are not identical.

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